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Restoration of the high-potential form of cytochrome *b*-559 by electron transport reactions through Photosystem II in Tris-treated Photosystem II membranes

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Abstract

The conversion of cytochrome (Cyt) *b*-559 from the low-potential (LP) to the high-potential (HP) form under conditions for photoactivation of O_2 evolution (reconstitution of the Mn cluster) was investigated using Photosystem II (PS II) membranes that had been depleted of the Mn cluster by treatment with Tris. Illumination of the PS II membranes with continuous or flashing light in the presence of 0.1 mM Mn^{2+} reactivated O_2 evolution and increased the level of the HP form of Cyt *b*-559 with a concomitant decrease in the level of the LP form. When illumination was achieved with flashing light, the restoration of the HP form occurred after two flashes, while reactivation of O_2 evolution required more than six flashes. It was also found that the HP form could be restored when the PS II membranes were illuminated in the presence of artificial electron donors instead of Mn^{2+}. NH_2OH (10–100 μM), 1,5-diphenylcarbazide (50–100 μM) and semicarbazide (0.5–1 mM) were effective in restoring the HP form. These observations suggest that, under photoactivation conditions, not the reconstitution of the Mn cluster but electron donation by Mn^{2+} to PS II is responsible for the restoration of the HP form. The restoration of the HP form by illumination in the presence of Mn^{2+} was not affected by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) but it was completely suppressed by artificial electron acceptors which bind to the Q_B site and reoxidize Q_A^-, namely, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB) and 2,6-dichloro-1,4-benzoquinone. These results suggest that some redox reaction(s) at the acceptor side of PS II, which probably involves Q_A^-, occurs during the course of the restoration of the HP form.

Keywords: Cytochrome *b*-559; Oxygen evolution; Photoactivation; Photosystem II

Abbreviations: Chl, chlorophyll; Cyt, cytochrome; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DCBQ, 2,6-dichloro-1,4-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DPC, 1,5-diphenylcarbazide; HP, high-potential; LP, low-potential; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Q_A and Q_B, primary and secondary quinone acceptors, respectively, of Photosystem II; VLP, very-low-potential; Y_Z, redox-active tyrosine-161 of the D1 protein.

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1. Introduction

Photosystem II (PS II), a membrane protein complex that is embedded in the thylakoid membrane, carries out the photochemical reaction and the subsequent electron-transport reactions from water to plastoquinone molecules [1]. The redox components involved in these reactions are all bound to the reaction center complex, which consists of a heterodimer of two homologous proteins, the D1 and D2 proteins [1]. The reaction center complex of PS II is considered to have a structure homologous to that of purple photosynthetic bacteria [2,3], but it differs from the bacterial complex in some respects. One major difference is that the PS II reaction center complex generates a high oxidizing potential of about +1.1 V and oxidizes water to evolve oxygen molecules [1]. The O_2-evolving reaction is catalyzed by the Mn cluster, which is bound to the complex at the lumenal surface of the thylakoid membrane [4]. Another difference is that a *b*-type cytochrome, Cyt *b*-559, is tightly bound to the PS II reaction center complex [5].

Cyt *b*-559 consists of two protein subunits, α and β, and it is considered that the heme iron forms cross-links with single histidine residues in each subunit [6]. From the orientation in the thylakoid membrane of each subunit, it has been suggested that the heme is located close to the stromal surface of the membrane [7,8]. It is unclear whether the PS II reaction center complex binds one or two molecules of Cyt *b*-559. In isolated thylakoids, it has been reported that two hemes of Cyt *b*-559 are present per PS II [9,10]. In the case of isolated PS II membranes, by contrast, conflicting results have been reported, namely, one heme [11–13] or two hemes [14,15] per PS II. The physiological role of Cyt *b*-559 also remains to be unequivocally resolved, and many possible roles for this cytochrome have been proposed (for reviews, see Refs. [16,17]). It has been suggested (1) that Cyt *b*-559 participates in the light-dependent reconstitution of the Mn cluster [16]; (2) that it protects PS II from photoinhibition by mediating a cyclic electron-transport reaction around PS II [18] or by oxidizing the reduced form of the primary electron acceptor, pheophytin, to suppress the overreduction of Q_A [19]; and (3) that it acts as an anchor during the assembly of the PS II reaction center complex [17].

One unique characteristic of Cyt *b*-559 is that its redox potential is variable, and it exists in three forms with different redox potentials, namely, the high-potential (HP), low-potential (LP) and very-low-potential (VLP) forms. The redox potentials of the HP, LP and VLP forms are about +370 mV, +60 mV and $\leq$ 0 mV, respectively [20]. An intermediate-potential form ($E'_o = +230$ mV) has also been reported [10]. In general, about half or more of Cyt *b*-559 exists in the HP form in materials in which PS II contains the Mn cluster and is active in O_2 evolution: about half of Cyt *b*-559 is in the HP form in vivo [21], in intact chloroplasts [22] and in isolated thylakoids [10,23], while, in isolated PS II membranes, the proportion of the HP form ranges from 50 to 90% of the total depending on the preparation [10,24,25].

The different potential forms of Cyt *b*-559 are partly interconvertible. The HP form can be converted to lower-potential forms in vitro when the O_2 evolution is inactivated by treatment with concentrated Tris, $CaCl_2$ or NaCl, which removes the Mn cluster and/or the extrinsic proteins that participate in the O_2 evolution [10,26,27]. This phenomenon is also observed in vivo: almost all Cyt *b*-559 exists in the LP and/or VLP form in materials in which PS II lacks in the Mn cluster and is inactive in O_2 evolution, as in the LF1 mutant cells of *Scenedesmus* [28], in wheat seedlings grown under intermittent illumination [29], and in dark-grown pine cotyledons [21]. Conversely, the HP form of Cyt *b*-559 can be restored when O_2 evolution is reactivated by reconstitution of the Mn cluster in PS II in vivo [21] and also in vitro [23,30].

From the correlation between the presence of the HP form and the capacity for evolving oxygen, it would appear that the binding of the extrinsic proteins and/or the Mn cluster to PS II might be necessary for retention of the HP form of Cyt *b*-559. However, recent studies have demonstrated that Cyt *b*-559 can remain in the HP form under some certain conditions, even after the extrinsic proteins and/or the Mn cluster have been removed from PS II [25,31,32]. Thus, unidentified factors appear to regulate the redox potential of Cyt *b*-559.

In this study, we investigated in detail the restoration of the HP form of Cyt *b*-559 during illumination in the presence of exogenous Mn^{2+}, the conditions that lead to the reconstitution of the Mn cluster (photoactivation of O_2 evolution), in PS II membranes that had been depleted of the three extrinsic proteins and the Mn cluster by treatment with Tris. We found that it was not the reconstitution of the Mn cluster but the electron donation by Mn^{2+} to PS II that was responsible for the restoration of the HP form. We found, furthermore, that electron donors other than Mn^{2+} were also effective in restoring the HP form during illumination. A possible mechanism for the change in the redox potential of Cyt *b*-559 is discussed.

2. Materials and methods

2.1. Preparation of thylakoids, PS II membranes and extrinsic proteins

Thylakoids were prepared from spinach leaves, treated with Tris and, subsequently, with reduced DCPIP, before they were subjected to the photoactivation treatment [23]. PS II membranes were prepared from spinach leaves as described by Kuwabara and Hashimoto [33] with the exception that a ratio of Triton X-100 to Chl of 15 (w/w) was used. The PS II membranes were suspended in 30% (v/v) ethylene glycol/20 mM NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5) and stored at −80°C. Before use, the PS II membranes were thawed on ice and washed three times with 20 mM NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5; low-salt medium) by centrifugation and resuspension. To remove the Mn cluster and the three extrinsic proteins from PS II, the PS II membranes were suspended in 0.8 M Tris-HCl (pH 9.1)/5 mM EDTA/2 mM sodium ascorbate at 0.5 mg Chl/ml and incubated in darkness for 2 h. The Tris-treated PS II membranes were collected by centrifugation at $35\,000 \times g$ for 20 min and washed twice with the low-salt medium by resuspension (0.3 mg Chl/ml) and centrifugation. They were finally suspended in the same medium supplemented with 30% (v/v) ethylene glycol and stored at −80°C. Before use, the Tris-treated PS II membranes were thawed and washed three times with the low-salt medium by centrifugation and resuspension. Handling of the Tris-treated PS II membranes was performed under dim light.

The extrinsic proteins of 33, 23 and 18 kDa were prepared from the Tris extract of PS II membranes. The supernatant after centrifugation that followed the treatment with Tris, prepared as described above, was dialyzed against 10 mM Mes-NaOH (pH 6.5) for 8 h and concentrated with a Centriprep 10 system (Amicon, USA). No contamination by other proteins was detected by SDS-polyacrylamide gel electrophoresis. All procedures were performed at 0–4°C. Chl concentration was determined as described by Arnon [34].

2.2. Photoactivation of O_2 evolution

Photoactivation of Tris-treated thylakoids was performed as described previously [23]. Photoactivation of Tris-treated PS II membranes was performed at 25°C by the method of Miyao and Inoue [35] with some modifications, as follows. Tris-treated PS II membranes, which had been kept in darkness at 0°C for more than 4 h, were suspended in 1 M NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5; high-salt medium). Final Chl concentrations were 125 and 250 μg/ml for photoactivation with continuous and flashing light, respectively. The suspension (6 and 1 ml for photoactivation with continuous and flashing light, respectively) was placed in a flat glass dish of 30 mm in diameter. Final thicknesses of the suspension were 8.6 and 1.4 mm for photoactivation with continuous and flashing light, respectively. After incubation in darkness at 25°C for 5 min, the suspension was supplemented with 0.1 mM $Mn(CH_3COO)_2$, 50 mM $CaCl_2$ and, when indicated, 2 μM DCPIP, and further incubated in darkness at 25°C for 5 min. Then, the suspension was illuminated from above with continuous light from a white fluorescent lamp at 33 $\mu E \cdot m^{-2} \cdot s^{-1}$ or it was illuminated with flashing light from a 10-μs xenon flash lamp (PS-240; Sugawara, Japan), with gentle stirring. Saturation of the light intensity of the flash was confirmed by use of a neutral-density filter (75%). After illumination, 50 μl of the photoactivation mixture was diluted 20-fold with the low-salt medium that contained the 33-, 23- and 18-kDa proteins at levels sufficient to maximize the O_2-evolving activity. The O_2-evolving

activity was measured at 20°C with 0.5 mM phenyl-1,4-benzoquinone as an artificial electron acceptor using a Clark-type oxygen electrode. Another portion of the photoactivation mixture was subjected to the analysis of Cyt *b*-559.

2.3. Analysis of Cyt b-559

Levels of Cyt *b*-559 in the different potential forms were determined from reduced-minus-oxidized difference spectra recorded with a spectrophotometer (UV-2200; Shimadzu, Japan), essentially as described previously [23]. When Tris-treated PS II membranes had been photoactivated with continuous light, the photoactivation mixture was directly subjected to analysis. When photoactivation was achieved with flashing light, the photoactivation mixture was diluted 5-fold with the high-salt medium that contained 0.1 mM $Mn(CH_3COO)_2$ and 50 mM $CaCl_2$ prior to the analysis. These concentrations of Mn^{2+} and Ca^{2+} and 2 μM DCPIP did not interfere with the analysis of Cyt *b*-559. For the reduction of Cyt *b*-559, the sample was supplemented sequentially with 2 mM hydroquinone ($E'_o = +290$ mV), 5 mM sodium ascorbate ($E'_o = +60$ mV) and a few mg of sodium dithionite. After each addition, the absorbance spectrum was recorded at the scanning rate of 100 nm/min with the slit width of 2 nm. The amounts of Cyt *b*-559 reduced by addition of hydroquinone (HP form), sodium ascorbate (LP form) and sodium dithionite (VLP form) were estimated from the difference spectra, with the absorbance spectrum recorded in the presence of 0.5 mM potassium ferricyanide taken as the reference spectrum. The half width of the band of reduced Cyt *b*-559 in the difference spectrum was 12 ± 0.5 nm and it was unchanged upon treatment with Tris and subsequent photoactivation treatment. A difference extinction coefficient [$\epsilon_{(559-570\ nm)}$] of 15 $mM^{-1} \cdot cm^{-1}$ [20] was used. The deviation of results was less than 5%.

3. Results

3.1. Changes in levels of the different potential forms of Cyt b-559 upon treatment with Tris and subsequent photoactivation treatment

The PS II membranes used in this study contained about two molecules of Cyt *b*-559 per 220 Chl molecules, a PS II unit. The levels of the different forms of Cyt *b*-559 in these PS II membranes were 1.0–1.2, 0.2–0.1 and 0.8–0.6/220 Chl (mol/mol) for the HP, LP and VLP forms, respectively. Treatment with Tris of the PS II membranes decreased the level of the HP form only by 0.3–0.4/220 Chl with a concomitant increase in the level of the LP form. The level of the VLP form remained unchanged. We

Table 1
Changes in the amount of the HP form of Cyt *b*-559 and in the O_2-evolving activity of Tris-treated PS II membranes upon photoactivation treatment

Additives	Cyt *b*-559 HP/220 Chl (mol/mol)			O_2 evolution after illumination (μmol · (mg Chl)$^{-1}$ · h^{-1})	
	Before illu.	After illu.	Δ	$-Ca^{2+}$	$+Ca^{2+}$
None	0.60	0.38	−0.22	8	–
Mn^{2+}	0.50	0.63	+0.13	8	47
Ca^{2+}	0.50	0.46	−0.04	8	–
Mn^{2+}, Ca^{2+}	0.50	0.70	+0.20	92	–
Mn^{2+}, Ca^{2+}, DCPIP	0.54	0.73	+0.19	172	–

Tris-treated PS II membranes were suspended in 1 M NaCl/0.4 M sucrose/50 mM Mes-NaOH (pH 6.5; high-salt medium) and incubated in darkness for 5 min. The suspension was supplemented with the indicated additive(s), incubated in darkness for 5 min, and then illuminated with continuous light for 10 min. Final concentrations of additives were 0.1 mM, 50 mM and 2 μM for Mn^{2+}, Ca^{2+} and DCPIP, respectively. The amount of the HP form of Cyt *b*-559 was determined from the (hydroquinone)-(ferricyanide) difference absorbance spectrum. The total amount of Cyt *b*-559 was 1.94/220 Chl (mol/mol). The O_2-evolving activity was measured in the presence of the three extrinsic proteins. When Ca^{2+} had been absent during illumination, 10 mM $CaCl_2$ was added to the assay mixture. The O_2-evolving activity of PS II membranes before Tris treatment was 405 μmol · (mg Chl)$^{-1}$ · h^{-1}.

confirmed by atomic absorption spectroscopy and SDS-polyacrylamide gel electrophoresis that the treatment with Tris had completely removed Mn and the three extrinsic proteins from the PS II membranes (data not shown). These observations are consistent with results of previous studies [25,31,32] that the removal of the Mn cluster and the extrinsic proteins does not necessarily cause the change in redox potential of Cyt *b*-559.

We investigated experimental conditions that would further lower the level of the HP form of Cyt *b*-559 in the Tris-treated PS II membranes. We found that incubation of the membranes with 1 M NaCl in darkness lowered the level by 0.1–0.4/220 Chl to give a final level of 0.3–0.6/220 Chl. Besides NaCl, NaF, NaBr and sodium formate at 1 M were each effective in lowering the level of the HP form, while 1 M Na_2SO_4 was ineffective. Thus, some factor other than the ionic strength appeared to affect the level of the HP form. The use of 1 M NaCl had another advantage in the present study since 1 M NaCl has been shown effectively to enhance the photoactivation of O_2 evolution in PS II membranes [35]. Therefore, to investigate the effects of the photoactivation on the redox potential of Cyt *b*-559, we performed photoactivation treatment in the presence of 1 M NaCl.

Table 1 shows the changes in the amount of the HP form of Cyt *b*-559 in Tris-treated PS II membranes by photoactivation treatment with continuous light. When the membranes were illuminated in the absence of Mn^{2+} and Ca^{2+}, the level of the HP form decreased by about 0.2/220 Chl. This decrease probably resulted from photoinhibition of PS II that had been depleted of the Mn cluster [31,36]. When the membranes were illuminated in the presence of Mn^{2+}, the level of the HP form increased by 0.13/220 Chl. The additional presence of Ca^{2+} during illumination enhanced the increase in the level of the HP form and about 0.2/220 Chl of the HP form was restored. DCPIP did not further affect the increase in the level of the HP form. On the other hand, illumination in the presence of Ca^{2+} alone did not increase the level of the HP form, even though Ca^{2+} suppressed the decrease in the level of the HP form during illumination. When the level of the HP form increased, the level of the LP form decreased concomitantly, while the level of the VLP form remained unchanged (data not shown). These results suggest that the LP form of Cyt *b*-559 is converted to the HP form by illumination in the presence of Mn^{2+}. The O_2-evolving activity also increased when the membranes were illuminated in the presence of Mn^{2+} (Table 1). The recovery of activity, however, was unrelated with the extent of the restoration of the HP form.

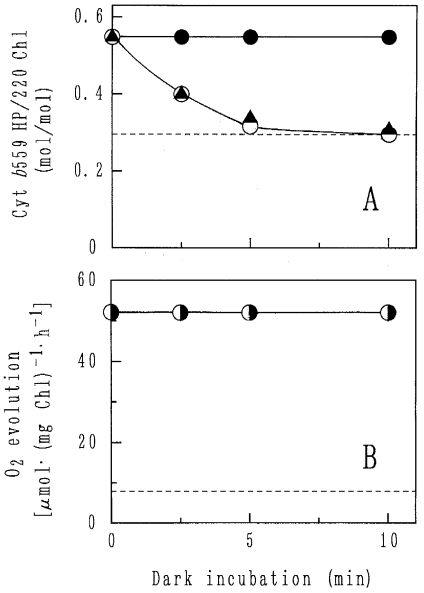

Fig. 1. Effects of Ca^{2+} on the stability of the HP form of Cyt *b*-559 and of the O_2-evolving activity which had been restored by photoactivation treatment. Tris-treated PS II membranes, suspended in the high-salt medium, were illuminated with continuous light in the presence of 0.1 mM Mn^{2+} for 10 min. After illumination, the suspension was incubated in darkness with no addition (○), with 50 mM $CaCl_2$ (●) or with 50 mM $MgCl_2$ (▲) for the designated times. (A) The amounts of HP form of Cyt *b*-559. The total amount of Cyt *b*-559 was 1.96/220 Chl. (B) O_2-evolving activity measured in the presence of 10 mM Ca^{2+}. The O_2-evolving activity of PS II membranes before treatment with Tris was 480 μmol·(mg Chl)$^{-1}$·h^{-1}. Broken lines represent the levels before illumination.

The photoactivation treatment and the subsequent analysis of Cyt *b*-559 in this study were performed in the absence of the three extrinsic proteins. Thus, it is suggested that the restoration of the HP form of Cyt *b*-559 does not require these extrinsic proteins. The extent of the restoration of the HP form varied among preparations, ranging from 0.15 to 0.35/220 Chl when the membranes were illuminated in the presence of Mn^{2+} and Ca^{2+}.

Fig. 1 shows the effects of Ca^{2+} on the HP form of Cyt *b*-559 that had been restored by the photoacti-

vation treatment. In this experiment, the Tris-treated PS II membranes were illuminated in the presence of Mn^{2+} alone and then they were incubated in darkness in the presence or in the absence of Ca^{2+}. It is obvious that the level of the HP form that had been restored by illumination remained unchanged in the presence of Ca^{2+}, while in the absence of Ca^{2+} the level decreased within 10 min to the level before illumination (Fig. 1A). Mg^{2+} did not have such stabilizing effects. By contrast, the O_2-evolving activity that had been restored by illumination remained unchanged during incubation in darkness both in the presence and in the absence of Ca^{2+} (Fig. 1B). These observations suggest that Ca^{2+} stabilizes the HP form of Cyt *b*-559 that had been restored by photoactivation treatment and that this stabilizing effect does not result from the stabilization by Ca^{2+} of the reconstituted Mn cluster. Ca^{2+} also appeared to stabilize the HP form of Cyt *b*-559 that had originally been present in the Tris-treated PS II membranes since the presence of Ca^{2+} suppressed the decrease in the level of the HP form during illumination in the absence of Mn^{2+} (Table 1). Thus, the stabilizing effects of Ca^{2+} appear to be independent of the presence of the Mn cluster.

Fig. 2 shows the restoration of the HP form of Cyt *b*-559 by photoactivation treatment with flashing light. Illumination with flashes in the presence of Mn^{2+} and Ca^{2+} restored both the HP form and the O_2-evolving activity, but the two phenomena exhibited quite different dependence on the flash number. The level of the HP form was unchanged by illumination with a single flash but it greatly increased after two flashes and reached a maximum with three flashes (Fig. 2A,B). By contrast, the recovery of O_2-evolving activity was detected only after illumination with six flashes and reached a maximum after 100 flashes (Fig. 2B). These observations indicate that the restoration of the HP form of Cyt *b*-559 is completed before the Mn cluster is reconstituted. It is also suggested that the HP form might be restored by two photoreactions in PS II.

Fig. 2B also shows the changes in the level of Cyt *b*-559 that remained in the reduced form after illumination. In general, the HP form can remain in the reduced form under aerobic conditions, while the lower-potential forms are, if reduced, readily reoxidized by ambient oxygen molecules. Therefore, in the

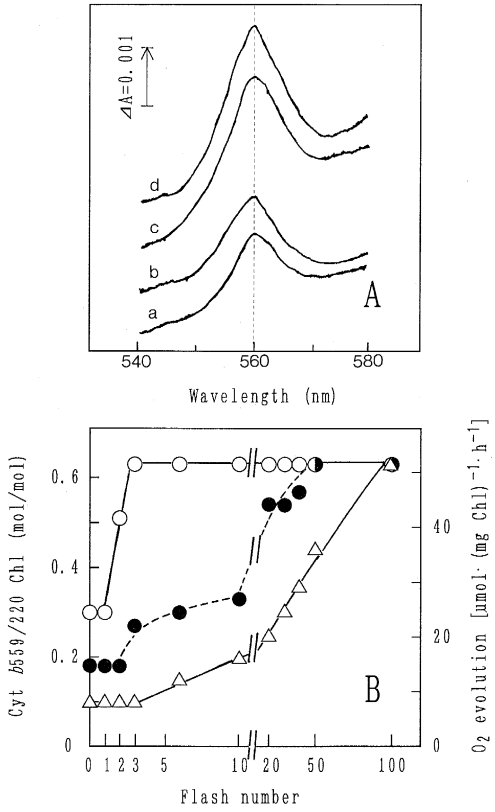

Fig. 2. Restoration of the HP form of Cyt *b*-559 and reactivation of O_2 evolution by photoactivation treatment with flashing light. Tris-treated PS II membranes, suspended in the high-salt medium, were illuminated with the designated numbers of flashes at intervals of 1 s in the presence of 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. (A) Changes in the (hydroquinone)-(ferricyanide) difference spectrum of Cyt *b*-559. (a) Before illumination; (b, c, d) after illumination with one, two and three flashes, respectively. (B) Changes in the amount of the HP form of Cyt *b*-559 (○), in the amount of Cyt *b*-559 that remained in the reduced form after illumination (●), and in the O_2-evolving activity (△). The total amount of the Cyt *b*-559 was 1.8/220 Chl. The O_2-evolving activity of PS II membranes before Tris treatment was 450 μmol·(mg Chl)$^{-1}$·h^{-1}.

experiment of Fig. 2B in which the absorption spectrum of the photoactivation mixture was recorded about two minutes after illumination, only the reduced HP form could be detected. The results indicate that the level of the reduced HP form was unchanged with one and two flashes but it gradually increased after three flashes. After illumination with 50 flashes, all the HP form was in the reduced form. Two interpretations can be considered for the gradual increase in the level of the reduced HP form: the

quantum efficiency of the reduction of the HP form was low and 50 flashes were required to reduce all the HP form, or the HP form was, once reduced, reoxidized by some reason and had to be reduced repeatedly for the accumulation of the reduced form.

To examine these possibilities, the change in the level of the reduced Cyt *b*-559 during illumination with flashes was followed by direct measurement of the absorbance change at 559 nm of the photoactivation mixture (Fig. 3). The absorbance change at 570 nm was also measured to follow the change in the base line of the absorption band of Cyt *b*-559. As seen in Fig. 3, the level of Cyt *b*-559 in the reduced form gradually increased during illumination with increasing number of flashes. After illumination with 10 flashes, however, the level of the reduced Cyt *b*-559 gradually decreased in darkness for about 30 s. On the other hand, after illumination with 40 flashes, the level of the reduced Cyt *b*-559 did not decrease but remained almost constant in darkness. In this experiment, a fraction of Cyt *b*-559 which was once reduced but reoxidized within 1 s could not be detected because of the limitation of time resolution of the instrument. Provided that only the reduced HP form was detected in this experiment, it is suggested that the reduced HP form was first unstable and susceptible to reoxidation but that it became more stable toward reoxidation by further illumination.

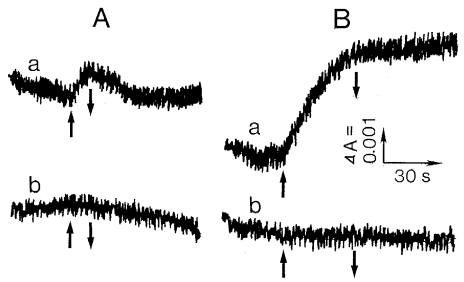

Fig. 3. Changes in the level of Cyt *b*-559 in the reduced form during and after photoactivation treatment with flashing light. Tris-treated PS II membranes were suspended in the high-salt medium that contained 0.1 mM Mn^{2+} and 50 mM Ca^{2+} at 100 μg Chl/ml and placed in a plastic cuvette (light-path length, 10 mm; light-path width, 4 mm). The cuvette was set in a spectrophotometer (UV-2200, Shimadzu) and the suspension was illuminated from the side of the cuvette with flashing light from a 10-μs xenon flash lamp, which had been passed through a red filter (R-62, Toshiba), at 1-s intervals. The photomultiplier was protected from red light with a blue filter (4-96, Corning). The absorbance of the suspension was recorded at 559 or 570 nm with the data-sampling interval of 0.1 s and the slit width of 5 nm. (A) Illumination with 10 flashes. (B) Illumination with 40 flashes. (a and b) Absorbance changes at 559 and 570 nm, respectively. Upward and downward arrows indicate the beginning and the end of illumination with a train of flashes, respectively. The absorbance change at 559 nm of 0.001 corresponds to 0.13 molecule of the reduced Cyt *b*-559 per 220 Chl molecules. The averages of five different experiments are presented.

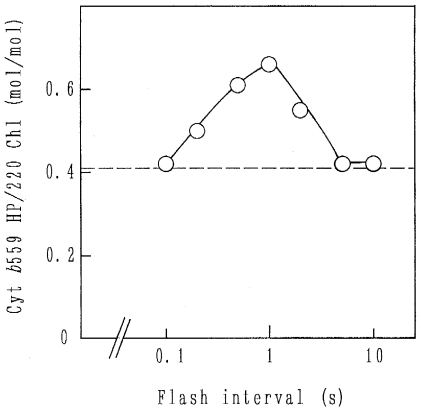

Fig. 4. Dependence on the flash interval of the restoration of the HP form of Cyt *b*-559 by photoactivation treatment with 5 flashes. Tris-treated PS II membranes, suspended in the high-salt medium, were illuminated with 5 flashes at the designated flash intervals in the presence of 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. The total amount of Cyt *b*-559 was 2.1/220 Chl. A broken line represents the level of the HP form before illumination. Three different experiments gave a similar dependence with a maximum restoration at 1-s intervals.

Fig. 4 shows the dependence on the flash interval of the restoration of the HP form. The dependence gave a bell-shaped curve, with the extent of the restoration was maximum at the interval of 1 s. If only one photoreaction were sufficient for the restoration, the extent of the restoration should increase with increasing intervals between flashes and then remain at a maximum plateau level at longer intervals. Therefore, it appeared that one photoreaction was insufficient for the restoration of the HP form. From this result and the dependence on the flash number in Fig. 2, it is suggested that two photoreactions are required for the restoration of the HP form.

The dependence on the flash interval also indicates that two photoreactions must occur successively for the restoration of the HP form. Illumination with flashes at intervals longer than 5 s was totally ineffective in the restoration (Fig. 4). This observation sug-

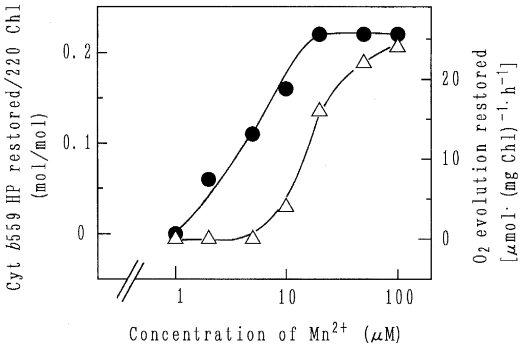

Fig. 5. Dependence on the concentration of Mn^{2+} during photoactivation treatment of the restoration of the HP form of Cyt *b*-559 and of the reactivation of O_2 evolution. Tris-treated PS II membranes, suspended in the high-salt medium, were illuminated with 30 flashes at intervals of 1 s in the presence of the designated concentrations of Mn^{2+} and 50 mM Ca^{2+}. The increases in levels of the HP form of Cyt *b*-559 (●) and O_2-evolving activity (△) upon illumination are presented. The amount of the HP form and the O_2-evolving activity before illumination were 0.47/220 Chl and 16 μmol·(mg Chl)$^{-1}$·h^{-1}, respectively.

gests that the restoration of the HP form proceeds via an unstable intermediary state which is generated after the first photoreaction but disappears in darkness within several seconds. The photoactivation of O_2 evolution yields a similar bell-shaped curve for the dependence on the flash interval [31,37]. However, the dependence of the restoration of the HP form in Fig. 4 was distinct from that for photoactivation since photoactivation treatment in this experiment was performed with five flashes, which had practically no reactivating effect on O_2 evolution (see Fig. 2B).

Fig. 5 shows the dependence on Mn^{2+} concentration of the restoration of the HP form and of the recovery of O_2-evolving activity upon the photoactivation treatment with 30 flashes. The HP form was effectively restored at Mn^{2+} concentrations below 10 μM, while the recovery of activity required much higher concentrations of Mn^{2+}. The concentrations that gave the half-maximal restoration were 5 and 17 μM for the HP form and the O_2-evolving activity, respectively. These differences in the requirement for Mn^{2+} again suggest that the restoration of the HP form of Cyt *b*-559 occurs independently of the reconstitution of the Mn cluster.

3.2. Factors involved in restoration of the HP form of Cyt b-559

As described above, the LP form of Cyt *b*-559 could be converted to the HP form by illumination with two flashes in the presence of Mn^{2+}. Two different roles for Mn^{2+} in this process can be considered: (1) Mn^{2+} could be essential, and the process could involve photooxidation of Mn^{2+} and binding of the resultant Mn^{3+} to the PS II reaction center complex, as in the case of the reconstitution of the Mn cluster [31,37]; or (2) Mn^{2+} could merely act as artificial electron donors and electron-transport reac-

Table 2
Changes in the amount of the HP form of Cyt *b*-559 upon illumination in the presence of artificial electron donors

Electron donor added	E'_o (mV)	Cyt *b*-559 HP/220 Chl (mol/mol)		
		Before illu.	After illu.	Δ
None		0.58	0.45	−0.13
100 μM Mn^{2+}	+1090	0.48	0.62	+0.14
1 mM NH_2OH	–	0.42	0.69	+0.27
1 mM semicarbazide	> +430	0.43	0.60	+0.17
100 μM DPC	+215–+430	0.42	0.63	+0.21
100 μM benzidine	+610	0.42	0.42	0
10 mM NaI	+560	0.50	0.27	−0.23

Tris-treated PS II membranes were suspended in the high-salt medium and incubated in darkness for 5 min. The suspension was supplemented with the indicated artificial electron donor, incubated in darkness for 5 min, and illuminated with continuous light for 10 min. The total amount of Cyt *b*-559 was 2.0/220 Chl. E'_o represents midpoint potentials taken from Clark [61]. For Mn^{2+}, the E'_o value of the Mn^{2+}/Mn^{3+} couple is presented [62]. For semicarbazide and DPC, the E'_o values were estimated to be higher than +430 mV and to be between +215 and +430 mV, respectively, since semicarbazide does not reduce ferricyanide (E'_o = +430 mV) and DPC reduces ferricyanide but not DCPIP (E'_o = +215 mV).

tions through PS II are responsible for the restoration of the HP form. We examined whether artificial electron donors other than Mn^{2+} could replace Mn^{2+}. As shown in Table 2, three of five artificial donors tested were effective in restoring the HP form of Cyt *b*-559 during illumination. NH_2OH (10–1000 μM), semicarbazide (0.5–1 mM) and DPC (50–100 μM) allowed restoration of the HP form more effectively than Mn^{2+}. Benzidine (10–100 μM) was ineffective, but it prevented any decrease in the level of the HP form during illumination. I^- did not restore the HP form but, in fact, tended to enhance the decrease in the level of the HP form. Identical results were obtained when Tris-treated PS II membranes which had been illuminated in the presence of artificial donors were washed twice with the low-salt medium to remove artificial donors prior to analysis of Cyt *b*-559 (data not shown).

The electron donors that were effective in the restoration of the HP form are all efficient electron donors to PS II [38–40]. Benzidine, which did not support the restoration, is also an efficient electron donor comparable to Mn^{2+} since benzidine and Mn^{2+} reduce Y_Z^+ with similar rate constants [41]. Thus, the rate of electron donation seems not to be correlated with the effectiveness in the restoration. The mid-point potentials of the artificial donors were also uncorrelated (Table 2). I^- is a poor electron donor. Illumination in the presence of I^- causes the specific iodination of Y_Z or the non-specific iodination of proteins of PS II in samples depleted of the Mn cluster, with resultant inactivation of electron transport in PS II [42,43]. These deleterious effects of I^- might be responsible for the decrease in the level of the HP form under illumination in the presence of I^-.

From the above observations, it is suggested that Mn^{2+} is not essential but electron donation by exogenous donors to PS II is responsible for the restoration of the HP form of Cyt *b*-559. We next investigated the events that occur at the acceptor side of PS II during the course of the restoration of the HP form.

Table 3 shows the effects of DCMU and benzoquinone acceptors on the restoration of the HP form of Cyt *b*-559 upon illumination in the presence of Mn^{2+} and Ca^{2+}. DCMU did not at all affect the restoration. This result suggests that electron transfer from Q_A to Q_B is not required for the restoration. By contrast, benzoquinone acceptors, namely, DCBQ and DBMIB, completely suppressed the restoration. This result contrasts with the effects of DCPIP which did not suppress the restoration (see Table 1). DCBQ and DBMIB bind to the Q_B site and directly oxidize Q_A^- [44,45], while DCPIP accepts electrons from Q_B and/or plastoquinone pool. Therefore, it seems likely that DCBQ and DBMIB had a suppressive effect when they were bound to the Q_B site. This possibility is supported by the observations that, when the Q_B site was first occupied by DCMU, further addition of DCBQ or DBMIB did not have a suppressive effect, while the addition of these compounds in the opposite order had a suppressive effect (Table 3). The most probable action of DCBQ and DBMIB in the Q_B site is to oxidize Q_A^-. Therefore, it seems likely that Q_A^- generated under illumination is necessary for the restoration of the HP form. It is also possible that DCBQ and DBMIB in the Q_B site affect some other redox component(s) in PS II which is involved in the restoration of the HP form.

Table 3
Effects of DCMU and artificial electron acceptors on the restoration of the HP form of Cyt *b*-559 by photoactivation treatment

Additives	Cyt *b*-559 HP/220 Chl (mol/mol)		
	before illu.	after illu.	Δ
None	0.47	0.69	+0.22
DCMU	0.50	0.71	+0.21
DCBQ	0.50	0.50	0
DBMIB	0.50	0.50	0
DCMU → DCBQ	0.50	0.66	+0.16
DCMU → DBMIB	0.50	0.74	+0.24
DCBQ → DCMU	0.50	0.50	0
DBMIB → DCMU	0.84	0.79	−0.05

Tris-treated PS II membranes were suspended in the high-salt medium, supplemented with 0.1 mM Mn^{2+} and 50 mM Ca^{2+} and incubated in darkness for 5 min. Then the suspension was further supplemented with the indicated additive(s), incubated in darkness for 2 min, and illuminated with five flashes at flash intervals of 1 s. The final concentration of each additive was 10 μM. When two reagents were added sequentially, the second reagent was added 1 min after addition of the first one (indicated by an arrow). The total amount of Cyt *b*-559 was 2.1/220 Chl.

We observed that, when DBMIB and DCMU were added sequentially, the level of the HP form increased even in darkness (Table 3). This restoration in darkness depended on the conditions of the dark adaptation of the Tris-treated PS II membranes prior to the experiment. When the membranes had been

adapted to darkness for 5 h on ice, the extent of the restoration in darkness was about 0.3/220 Chl. However, when the membranes had been dark-adapted at 25°C, the extent of the restoration decreased by about 50%. Thus, some redox power that remained in PS II appeared to be responsible for this restoration in darkness, via some as yet unexplained mechanism.

We proposed previously that, in Tris-treated thylakoids, the pH gradient across the thylakoid membrane might participate in the restoration of the HP form of Cyt *b*-559 upon the photoactivation treatment, since uncouplers, namely, nigericin and gramicidin-S at 100 μM, almost completely inhibited the restoration [23]. The PS II membranes used in the present study were not closed membrane vesicles but were membrane fragments [46] and, thus, no pH gradient could be formed across the membrane. We reinvestigated the effects of nigericin on the restoration of the HP form in Tris-treated thylakoids and Tris-treated PS II membranes (Fig. 6). At 10 μM, nigericin inhibited the restoration of the HP form by about 20% in the thylakoids but no inhibition at all was noted in the PS II membranes. At concentrations above 10 μM, nigericin had inhibitory effects in both cases, though the extent of inhibition in the PS II membranes was about 50% of that in the thylakoids at all tested concentrations of nigericin. In general, nigericin at 10 μM is sufficient to diminish the pH gradient across the thylakoid membrane. Therefore, it seems likely that, while the inhibition by nigericin at 10 μM observed in the thylakoids might be ascribable to disruption of the pH gradient, the inhibition at concentrations above 10 μM results from some additional effect of nigericin. It appears that a pH gradient across the membrane is not a prerequisite for the restoration of the HP form of Cyt *b*-559, even though such a gradient acts to enhance the restoration in thylakoids.

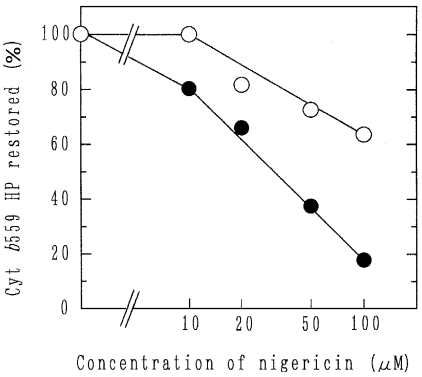

Fig. 6. Effects of nigericin on restoration of the HP form of Cyt *b*-559 by photoactivation treatment. Tris-treated PS II membranes (○) were suspended in the high-salt medium that contained 0.1 mM Mn^{2+} and 50 mM Ca^{2+}. Thylakoids that had been treated with Tris and then with DCPIP (●) were suspended in a medium that contained 50 μM Mn^{2+}, 1 mM Ca^{2+}, 20 μM DCPIP and 2 mM ascorbate [23]. Then the suspension was supplemented with designated concentrations of nigericin and 10 mM KCl, incubated in darkness for 2 min, and illuminated with continuous light. The duration of illumination was 10 and 20 min for the PS II membranes and the thylakoids, respectively. The amount of the HP form of Cyt *b*-559 in the thylakoids was determined after the thylakoids had been washed twice with a medium without Mn^{2+}, Ca^{2+} and DCPIP. 100% corresponds to the amount of the HP form of Cyt *b*-559 restored by photoactivation treatment in the absence of nigericin, namely, 0.22/220 Chl in the PS II membranes and 0.56/400 Chl in the thylakoids. The total amounts of Cyt *b*-559 were 2.1/220 Chl and about 2/400 Chl in the PS II membranes and the thylakoids, respectively.

4. Discussion

4.1. Interconversion of the LP and HP forms of Cyt b-559

Using isolated thylakoids, we demonstrated previously that the HP form of Cyt *b*-559 can be converted to the LP form by treatment with Tris and that the LP form can be recovered to the HP form by photoactivation of O_2 evolution [23,30]. The present study showed that the same interconversion of the LP and HP forms can also occur in isolated PS II membranes. However, the extent of interconversion was smaller in PS II membranes than in thylakoids.

Under the conditions employed in this study, only about 30–40% of the HP form in PS II membranes was converted to the LP form when the Mn cluster and the three extrinsic proteins had been completely removed by treatment with Tris. In the case of intact thylakoids, by contrast, about 80% of the HP form was readily converted to the LP form upon treatment with Tris [23]. To lower further the level of the HP form in the Tris-treated PS II membranes, we incubated the membranes with 1 M NaCl in darkness.

It was reported previously that Ca^{2+} bound to the donor side of PS II, possibly to the Mn cluster, is

released under illumination in the presence of concentrated NaCl, with resultant conversion of the HP form of Cyt *b*-559 to the lower-potential forms [25]. However, it is unlikely that the effects of 1 M NaCl observed in this study resulted from the release of Ca^{2+}, since the releasable Ca^{2+} at the donor side would have already been lost upon removal of the Mn cluster by treatment with Tris [47]. We consider that the effects of concentrated NaCl are ascribable to the chaotropic effects of Cl^-. It was proposed previously that Cyt *b*-559 exists in the HP form when the heme is located inside a hydrophobic niche that is shielded from water molecules by protein segments, while the HP form is converted to the LP form when the heme is exposed to the outer aqueous phase [48]. Since Cl^- is a chaotropic anion that destroys the water structure at the surface of proteins and membranes, it is likely that concentrated Cl^- alters the environment around the heme to allow water molecules access to the heme.

The extent of restoration of the HP form by photoactivation was also smaller in PS II membranes than in thylakoids: 30–50% and 50–80% of the total LP form were converted to the HP form in the PS II membranes and in the thylakoids, respectively. If the interconversion of the HP and LP forms were to result from changes in the hydrophobicity around the heme, it seems likely that protein segments that shield the heme of Cyt *b*-559 can undergo conformational change more easily in thylakoids than in PS II membranes upon treatment with Tris and subsequent photoactivation.

Under photoactivation conditions, illumination in the presence of Mn^{2+} was sufficient for the restoration of the HP form (Table 1). The extrinsic proteins and Ca^{2+} were not required for this process. However, we found that Ca^{2+} was necessary for stabilization of the HP form of Cyt *b*-559 and that this stabilizing effect was independent of the presence of the Mn cluster (Table 1, Fig. 1). A similar stabilizing effect of Ca^{2+} on the HP form was also proposed previously in the case of PS II membranes that contained the Mn cluster but lacked the extrinsic 23-kDa protein [25].

Intact PS II membranes contain two molecules of Ca^{2+} per PS II [49]. One of the two Ca^{2+} is released from PS II upon removal of the Mn cluster [47] and this Ca^{2+} is considered to interact with the Mn cluster [50]. The other Ca^{2+} is tightly bound to the light-harvesting Chl complex and is not releasable [51]. In the case of PS II membranes that contain the Mn cluster, it is possible that Ca^{2+} binds to its specific binding site close to the Mn cluster and stabilizes the HP form of Cyt *b*-559 as proposed previously [25]. By contrast, in the case of PS II membranes depleted of the Mn cluster, it is unclear if the specific binding site for Ca^{2+} remains intact. Provided that Ca^{2+} can bind to its specific binding site both in the presence and in the absence of the Mn cluster, the effects of bound Ca^{2+} on the HP form would appear to be conformational since the Ca^{2+}-binding site is located at the lumenal surface of the PS II reaction center complex, at a distance from the heme of Cyt *b*-559.

4.2. Mechanism of the restoration of the HP form of Cyt b-559

It was reported previously [52] that the HP form of Cyt *b*-559 was restored when isolated thylakoids that had been extracted with hexane were reconstituted with β-carotene and plastoquinone molecules. The restoration of the HP form was also observed when purified Cyt *b*-559 was incorporated into liposomes that consisted of digalactosyldiacylglycerol [53]. The restoration of the HP form in these cases would be ascribable to the effects of exogenously added hydrophobic molecules which could directly alter the hydrophobicity around the heme of Cyt *b*-559. In the case of the restoration of the HP form upon photoactivation, illumination of PS II in the presence of Mn^{2+} was sufficient for the restoration.

The results of the present study show clearly that the restoration of the HP form and the reconstitution of the Mn cluster are different processes. The restoration of the HP form occurred with high quantum efficiency and was completed before the Mn cluster was reconstituted (Fig. 2B). In addition, the concentration of Mn^{2+} required for the restoration was much lower than that for the reconstitution of the Mn cluster (Fig. 5). The only similarity between these two processes is that both require two successive photoreactions in PS II (Figs. 2 and 4) [31,37].

Artificial electron donors other than Mn^{2+} also supported the restoration of the HP form under illumination (Table 2). This finding clearly indicates that

Mn^{2+} is not essential while electron donation from exogenous donors to PS II is required for the restoration of the HP form.

We also found that some redox component(s) that interacts with benzoquinone acceptors in the Q_B site participates in the restoration of the HP form (Table 3). The most probable candidate for this component is Q_A^- generated during illumination. According to Johnson et al. [54], upon removal of the Mn cluster, Q_A is converted from an active form with a low potential ($E'_o = -80$ mV) to an inactive form with a high potential ($E'_o = +55$ mV), which is unable to transfer electrons to Q_B. When Q_A is in the inactive form, Q_A^- generated by a photoreaction in PS II decays back to Q_A as a consequence of a charge recombination reaction with Y_Z^+, with a half time of 120 ms, unless Y_Z^+ is not re-reduced by exogenous donors [55]. If Q_A^- were to participate in the restoration of the HP form of Cyt *b*-559, it might be possible that the role of exogenous electron donors in the restoration is to stabilize Q_A^- by reducing Y_Z^+ and suppressing the charge recombination reactions. In fact, it has been reported that the presence of exogenous donors extends the lifetime of Q_A^- to more than a few seconds [55,56].

If some redox component(s) other than Q_A^- could participate in the restoration, the non-heme iron at the acceptor side of PS II would be a likely candidate. In general, the non-heme iron is in the Fe(II) state both in the light and in the dark, but it is oxidized under illumination in the presence of benzoquinone acceptors via the reduction-induced oxidation mechanism that involves oxidation of Q_A^- by a benzoquinone molecule bound to the Q_B site [57]. This oxidation of the non-heme iron does not occur when the Q_B site has been occupied by DCMU prior to addition of benzoquinone acceptors [57]. This effect of the sequential addition of DCMU and benzoquinone acceptors on the oxidation of the non-heme iron is quite similar to that on the restoration of the HP form (Table 3). Since benzoquinone acceptors completely suppressed the restoration (Table 3), it seems likely that the non-heme iron had to stay in the Fe(II) state during the course of the restoration of the HP form.

The most probable action of the putative redox component(s), Q_A^- and/or the non-heme iron, in the restoration of the HP form would be to reduce the LP form of Cyt *b*-559. It has been demonstrated recently that, in the case of horse heart Cyt *c*, the native protein in the reduced form is more stable toward unfolding than in the oxidized form, and that the reduction of the heme shifts the equilibrium from the denatured and low-potential form toward the native and high-potential form [58]. In the case of Cyt *b*-559, it is known that the HP form in the reduced form is more resistant to treatments which lower the redox potential of Cyt *b*-559 than in the oxidized form [25,59]. In addition, it was reported that the LP form of Cyt *b*-559 can be reduced by a photoreaction in PS II under some certain conditions [19,60]. Therefore, it is not unlikely that the LP form of Cyt *b*-559 is reduced during the course of the restoration of the HP form. If the LP form were to be reduced for the restoration, the reduced LP form appeared to be reoxidized within 1 s since, in the experiments in Fig. 3, significant reduction of Cyt *b*-559 was not detected after illumination with three flashes with which the restoration of the HP form was completed.

We observed that the HP form of Cyt *b*-559 restored by illumination with a few flashes remained in the oxidized form after illumination, but that the level of its reduced form increased by further illumination with flashes (Fig. 2B). We also found that the reduced HP form was first susceptible to reoxidation but became more stable toward reoxidation by further illumination (Fig. 3). Under the conditions in this study, not only the HP form of Cyt *b*-559 ($E'_o = +370$ mV) but also the intermediate-potential form(s) of the E'_o value higher than $+200$ mV could be detected as the HP form that was reducible with 2 mM hydroquinone (see Section 2). Therefore, it might be possible that the redox potential of the hydroquinone-reducible HP form restored with a few flashes was lower than that of the real HP form but increased gradually during further illumination so that the reduced form became stabilized. In fact, our preliminary experiments have shown that the hydroquinone-reducible HP form restored by illumination with two flashes was not reduced by 2 mM potassium ferrocyanide, while about 50% of the restored HP form was reduced by ferrocyanide after illumination with 40 flashes (data not shown). Thus, it is likely that the conversion of the LP form of Cyt *b*-559 to the real HP form proceeds via the intermediate-potential form(s), the reduced form(s) of which is unstable and gradually reoxidized by ambient oxygen molecules.

We presume that the LP form could be first converted to such an intermediate-potential form by two photoreactions in PS II, and the further conversion to the real HP form would require additional photoreactions in PS II. These photoreactions might lead to reduction of Cyt *b*-559 and shift the equilibrium toward higher-potential forms.

The dependence on the flash interval of the restoration of the hydroquinone-reducible HP form (Fig. 4) suggests that an unstable intermediary state would be formed between two photoreactions. The origin of this intermediary state is obscure. It also remains to be solved if the LP form had to be reduced twice for the conversion to the putative intermediate-potential form. Further studies are required to clarify the precise mechanism of the restoration of the HP form of Cyt *b*-559.

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